Appendix II
Chapter 3 Methods

General. Unless otherwise noted, reagents were purchased from the commercial suppliers Fisher (Fairlawn, NJ) and Sigma-Aldrich (St. Louis, MO), and were used without further purification. Protease and phosphatase inhibitors were purchased from Sigma-Aldrich or Alexis Biochemicals (San Diego, CA). Sequencing grade trypsin and chymotrypsin were obtained from Promega (Madison, WI) and Roche Applied Science (Indianapolis, IN), respectively. Sequencing grade endoproteinase Glu-C (protease V8) was purchased from Roche Applied Science (Indianapolis, IN). The MALDI matrix, α-cyano-4-hydroxycinnamic acid, was purchased from Fluka (Milwaukee, WI). Restriction enzymes were obtained from Fisher, with the exception of NdeI (Promega) and DpnI (New England Biolabs, Beverly, MA).

Purification of CREB and OGT from *Spodoptera frugiperda* (Sf9) and Hi-5 Insect Cells and *E. coli*. Rat CREB and human OGT cDNA clones were generously provided by Dr. R. H. Goodman (Oregon Health & Science University) and Dr. J. A. Hanover (NIDDK, National Institutes of Health), respectively, and were cloned into baculovirus expression vectors in frame with a histidine tag [1, 2]. Baculovirus preparation and protein expression in Sf9 cells were performed by Dr. P. Snow at the Beckman Institute Protein Expression Facility at the California Institute of Technology [3]. Proteins were purified using Ni-NTA agarose (Qiagen, Valencia, CA) with the following modifications to the manufacturer's protocol. For optimal solubility, the lysis, wash and elution buffers were supplemented with 10 mM BME and 0.1% Triton X-100. Purified CREB and OGT were

dialyzed into CREB storage buffer or OGT storage buffer (50 mM Tris-HCl pH 7.5, 12.5 mM $MgCl_2$, 40% glycerol) and stored at -80° C.

The relative levels of *O*-GlcNAc glycosylation were compared for CREB co-expressed in the absence and presence of OGT as follows. After labeling with GalT, CREB was resolved by SDS-PAGE. The gels were stained with Coomassie and treated with Amplify solution as described above. The relative glycosylation levels were measured by analysis of fluorography and Coomassie images using NIH Image 1.52 software. The data, which were normalized for CREB concentration, showed a 5.8-fold increase in the level of CREB glycosylation upon co-expression with OGT. As described below, similar values were obtained by LC-MS/MS analysis.

Purification of CREB from Rat Brain. CREB was purified from rat brain nuclear extracts by DNA affinity chromatography using minor modifications to published procedures [4]. The cAMP-Response Element (CRE) DNA affinity column was prepared as follows. Single-stranded oligonucleotides containing the CRE sequence and BamHI restriction sites (5'-GGATCCGCC**TGACGTCA**GAG-3' and 5'-GGATCCCTC**TGACGTCA**GGC-3'; the CRE sequence is shown in boldface) were annealed by combining equimolar amounts in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and incubating 2 min 88° C, 10 min 65° C, 10 min 37° C, 5 min 25° C and stored at 4° C. dsDNA was 5' phosphorylated with polynucleotide kinase (Roche Applied Sciences) in 50 mM Tris-HCl pH 8.2, 10 mM $MgCl_2$, 10 μM EDTA, 5 mM DTT, 0.1 mM spermidine, 3 mM ATP for 3 h at 37° C. The phosphorylated oligonucleotides (~40 μM) were then ligated with T4 DNA ligase (Roche Applied Sciences) in 66 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 4 mM ATP overnight at 16° C.

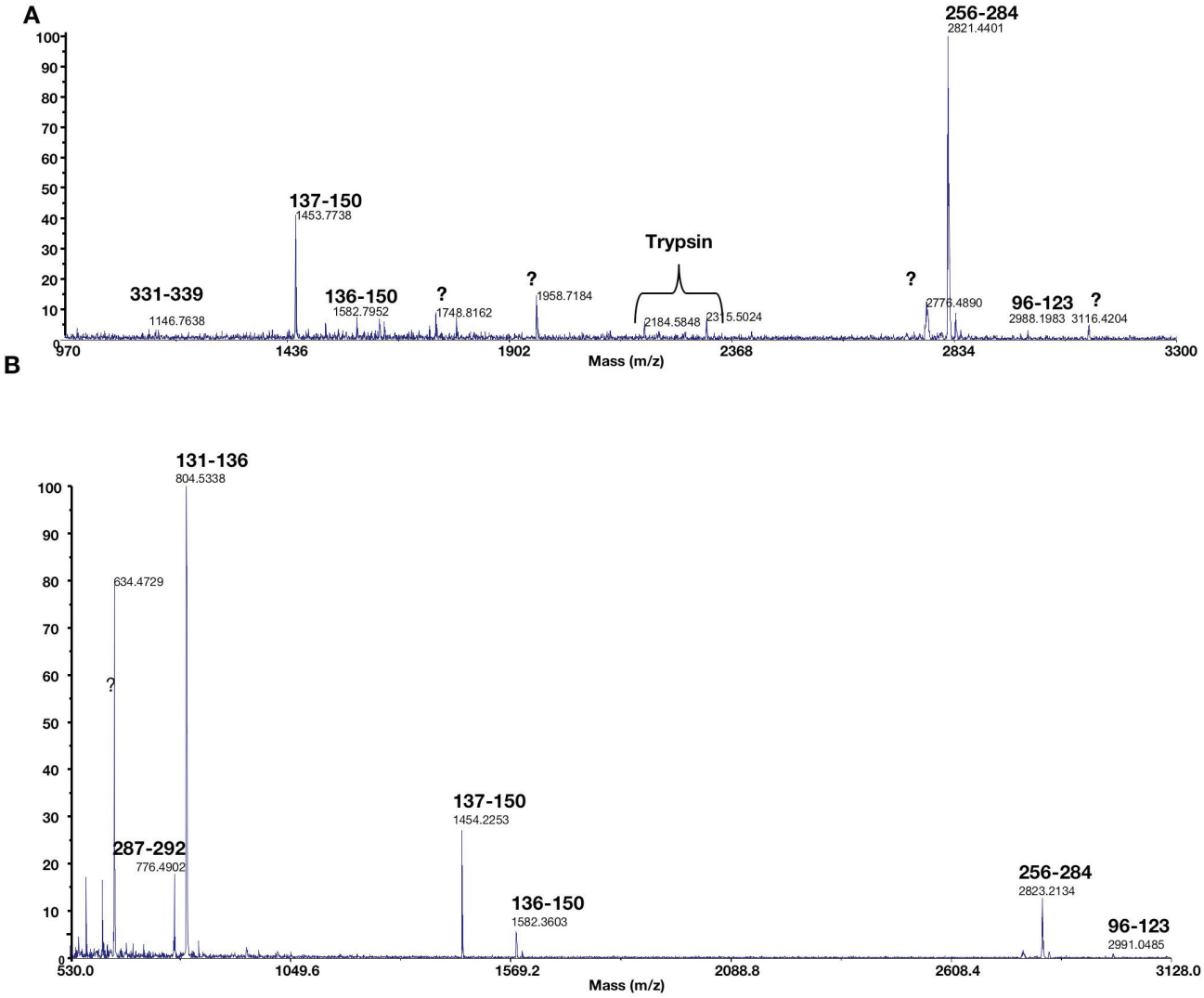
Following the ligation, the CRE oligonucleotides were precipitated with ethanol and resolubilized in the coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl; 2 mg/mL). Coupling of the oligonucleotides to CNBr-activated sepharose (Amersham Biosciences) was performed according to the manufacturer's protocol, using 4 mg of CRE oligonucleotides per mL of sepharose.

Nuclear extracts were prepared from 30 rat forebrains as described above and dialyzed into buffer E (50 mM Tris-HCl pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 20% (v/v) glycerol, 0.1% (v/v) NP-40). Extracts (2 mg/mL) were incubated with an equal volume of CRE affinity sepharose for 10 h at 4° C. The sepharose was washed with 5 volumes of buffer E, and proteins bound to the column were eluted with 2.5 volumes of buffer E containing 1 M KCl. Fractions enriched in CREB were identified by Western blot analysis, precipitated with 10% trichloroacetic acid/acetone and resolved by SDS-PAGE. The CREB protein was excised from the Coomassie-stained gels, digested with trypsin and analyzed by MALDI-TOF MS and LC-MS/MS.

Tryptic, Chymotryptic and Glu-C (V8) In-Gel Digests and MALDI-MS Analysis.

Tryptic digests were performed using methods developed by Dr. G. Hathaway at the Beckman Institute Protein/Peptide Microanalysis [5]. Digests with chymotrypsin and Glu-C were performed according to manufacturer's protocol (Roche, Indianapolis, IN). Briefly, recombinant CREB (1-4 μg) was resolved by SDS-PAGE, visualized by Coomassie Blue staining (0.1% Coomassie Brilliant Blue, 50% aqueous MeOH, 5% acetic acid) for 30 min at room temperature and destaining in 50% aqueous MeOH 5% acetic acid. CREB bands were

excised, reduced in 8 mM Tris(2-carboxyethyl)phosphine (TCEP), 80 mM NH_4HCO_3 pH 7.8, 50% aqueous CH_3CN for 20 min at room temperature, alkylated in 10 mM iodoacetamide, 50 mM NH_4HCO_3 pH 7.8, 50% aqueous CH_3CN for 20 min at room temperature in the dark, rehydrated in water and then dried on a Savant SpeedVac (GMI, Ramsey, MN). Proteolytic digests were resuspended in either trypsin (75-150 μl, 5-10 μg/ml in 50 mM NH_4HCO_3 pH 7.8), Chymotrypsin (75-150 μl, 1-2 μg/ml in 100 mM Tris-HCl pH 7.8, 10 mM $CaCl_2$) or Glu-C (75-150 μl, 10 μg/ml in 50 mM NH_4HCO_3 pH 7.8) and incubated overnight at 37^o C. Only enough protease solution was used to rehydrate the gel piece, usually 100-200 μl, and any excess liquid was removed. Peptides were extracted with 1x 100 μl water, 2x 200 μl 50% aqueous CH_3CN, 5% formic acid for 20 min/wash with constant agitation and concentrated by SpeedVac. For MALDI analysis, samples were concentrated on C18 zip tips (Millipore, Bedford, MA) and were combined with the MALDI matrix, 1 volume of saturated α-cyano-4-hydroxycinnamic acid in 50% aqueous CH_3CN with 0.1% TFA (Fluka, St. Louis, MO). All MALDI spectra were acquired on a PerSeptive Biosystems Voyager-DE Pro at 20,000 kV in either the reflector or linear mode. Samples were internally calibrated based on the monoisotopic *m/z* of tryptic peptides calculated using Protein Prospector v 4.0.4. CREB coexpressed in Sf9 cells with OGT exhibited identical fragmentation patterns to CREB expressed alone, although as anticipated, the intensities of the glycopeptide ions were lower in the absence of OGT. Appendix Figure 2 shows MALDI spectra acquired for CREB expressed in insect cells.



Appendix Figure 2. In-solution and in-gel trypsin digests of CREB. **(A)** In-gel trypsin digest of insect cell expressed CREB. **(B)** In-solution trypsin digest insect cell expressed CREB. Numbering indicates peptides that match predicted tryptic CREB peptides. Peaks labeled "Trypsin" are identified autoproteolytic peaks. CREB peptides were assigned based on an *in silico* digest performed with Protein Prospector on the UCSF web site (http://prospector.ucsf.edu).

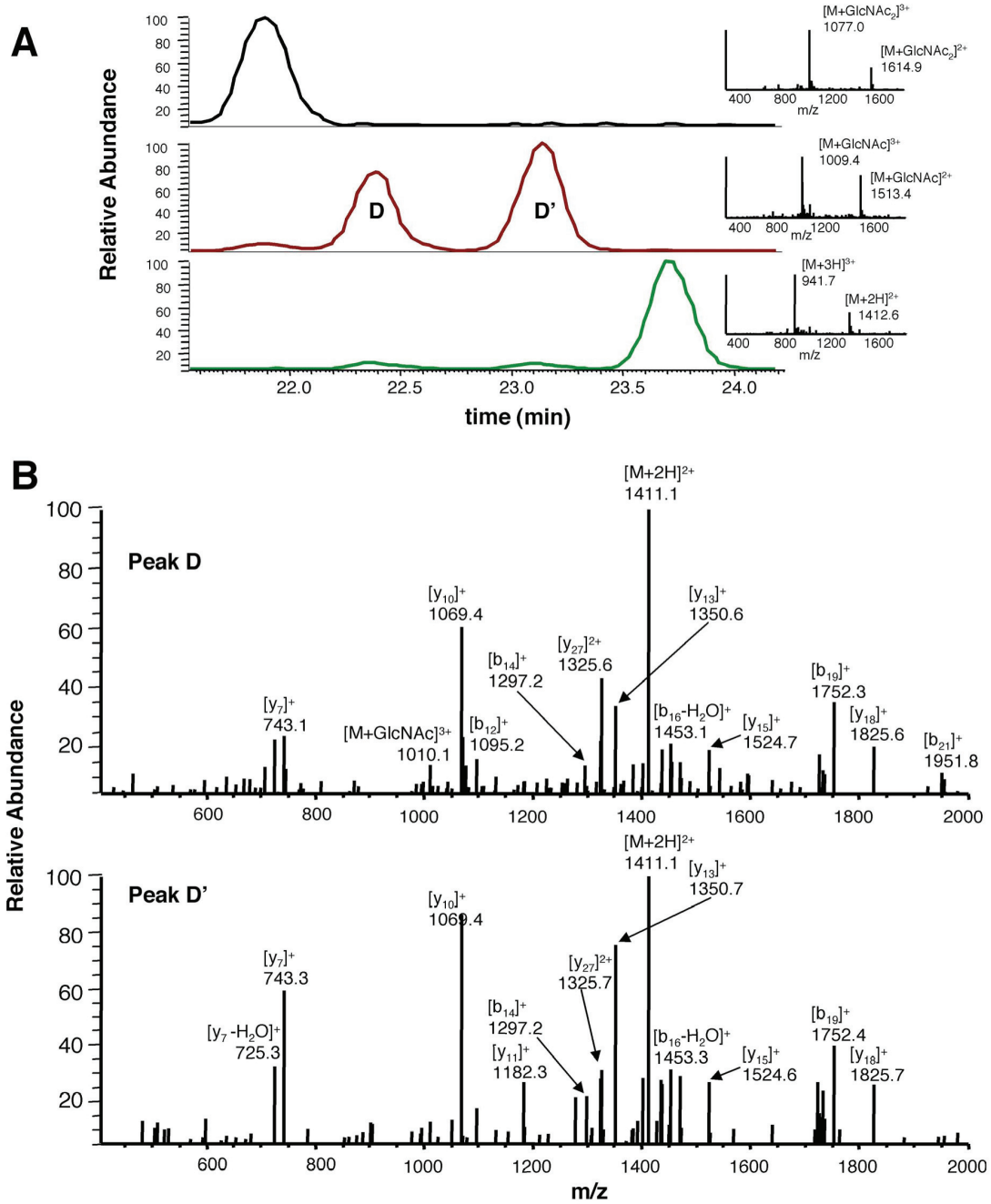
Cyanogen Bromide (CNBr) In-Gel Digests for MALDI-MS Analysis. CREB was resolved by SDS-PAGE and in-gel samples were prepared as described above. CNBr digest samples were treated according to the protocol of Vihinen and Saarinen [6]. Briefly, dehydrated gel samples were rehydrated in CNBr (5 mg/ml (Sigma-Aldrich, St Louis, MO) in 70% aqueous formic acid (Fluka, St. Louis, MO)) and incubated in glass vials, in the dark,

at room temperature for ~24 h. Only enough CNBr solution was used to rehydrate the gel piece, usually 100-200 μl, and any excess liquid was removed. Peptides were extracted and prepared for MALDI-ToF mass spectrometry as described above.

LC-MS/MS Analysis of Recombinant and Native CREB. Liquid chromatography and tandem mass spectrometry (LC-MS/MS) were performed on an LCQ Classic ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) that was fitted with a laboratory-built nanospray source and interfaced with a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). Tryptic digests of recombinant CREB were analyzed using a PicoFrit™ capillary column (0.075 mm i.d. x 50 mm; New Objective, Woburn, MA) packed with 5 μm 100 Å C18 reverse phase (RP) particles (Magic C18, Michrom BioResources, Auburn, CA). All other digests were analyzed using a Michrom BioResources capillary column (0.3 mm i.d. x 150 mm) pre-packed with 5 μm 200 Å C18 RP particles. To achieve microflow rates compatible with capillary chromatography (~1 μL/min), the flow from the HPLC was reduced with a splitting tee and a 150 mm section of fused silica tubing (0.05 i.d.). Approximately 2 pmoles of the tryptic digests were loaded on the microcolumns using the Surveyor Autosampler (ThermoFinnigan) and separated by RP chromatography. The LC buffers used were A: 2% CH_3CN, 0.1% AcOH, 0.005% heptafluorobutyric acid (HFBA), 97.9% H_2O and B: 90% CH_3CN, 0.1% AcOH, 0.005% HFBA, 9.9% H_2O. The gradient, which was optimized for separation of the glycosylated peptides, consisted of: 0-5 min, 0% B; 5-10 min, 0-18% B; 10-50 min, 18-40% B; 50-55 min, 40-100% B; 55-60 min, 100% B.

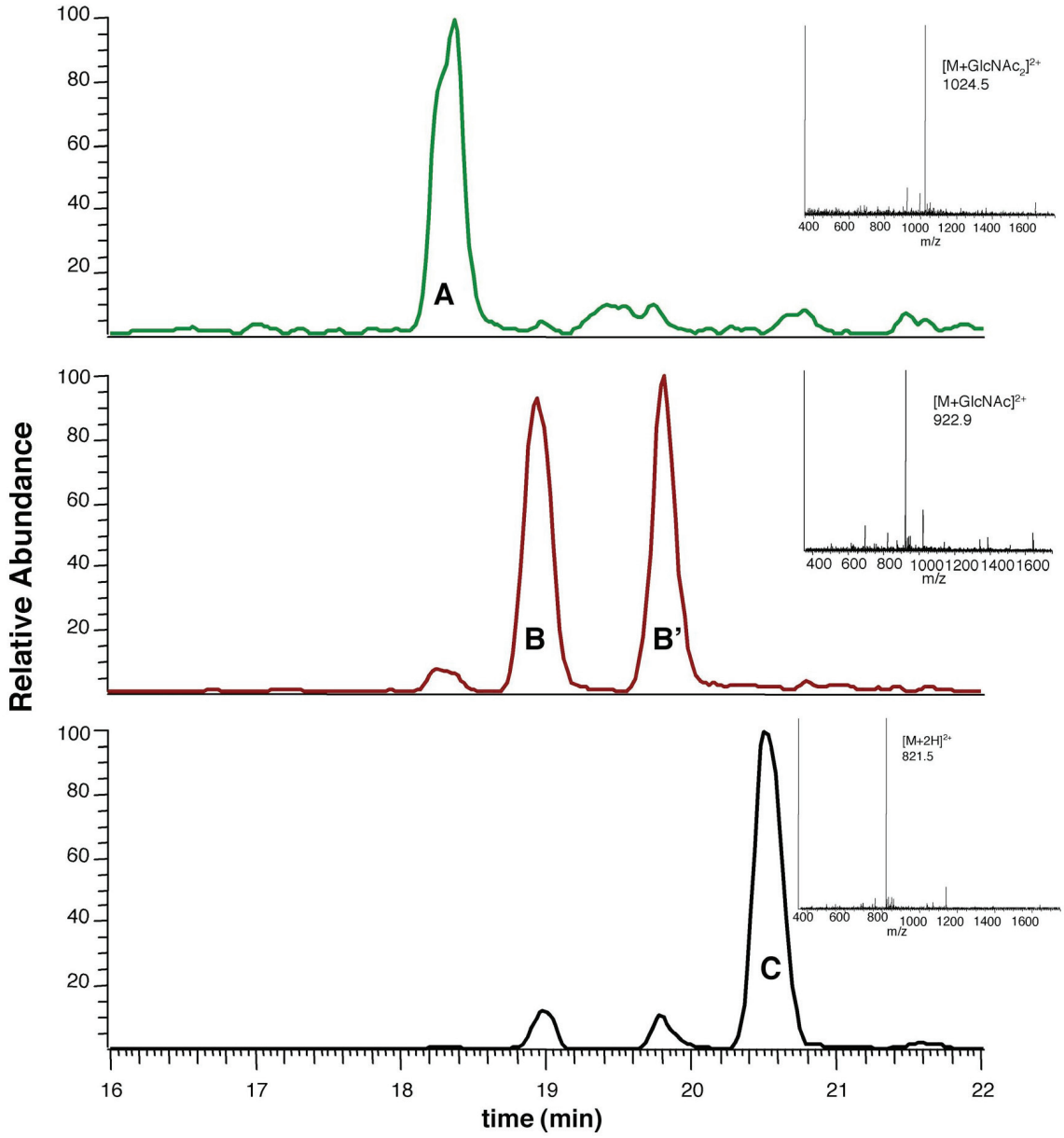
The LCQ was operated in automated mode using the Xcalibur™ software of the LCQ. The nanospray voltage was 1.8 kV and the heated capillary was 170-180˚ C. Automatic gain control was active, and the ion injection time was set at 200 ms for full scan mode of operation (3 scans per scan) or 400 ms (5 scans per scan) for MS/MS mode. Dynamic exclusion was used during data acquisition to ensure that the majority of co-eluting peaks would be selected for collision-induced dissociation (CID). In this mode of analysis, the ion trap acquires full scan MS spectra until an ion is present in a scan above a specified threshold. This triggers the ion trap to isolate that ion and generate a product ion spectrum (MS/MS). The ion trap returns to full scan operation when the ion intensity drops below the specified threshold. For native CREB, a parent ion of 1512.8 *m/z*, corresponding to $[M+GlcNAc]^{2+}$, was selected for MS/MS analysis. Relative collision energy for CID was preset to 35% and a default charge state of +2 was selected to calculate the scan range for acquiring tandem MS spectra. The precursor ion isolation window was set at 2.5 for maximum sensitivity.

Appendix Figure 3 shows selective ion chromatograms for four distinct forms of a tryptic peptide corresponding to residues 253-268 of CREB. As expected, sequential elution of the glycoforms was observed by reverse-phase LC, with the diglycosylated form eluting first, followed by the monoglycosylated forms (D and D') and then the unglycosylated peptide. Representative MS/MS spectra of the glycoforms D and D' ($[M+GlcNAc]^{2+}$ = 1513.4 *m/z*, where M = ^{256}TAPTSTIAPGVVMASSPALPTQPAEEAAR^{284}) are shown in Appendix Figure 3. The major ion observed after CID is the parent ion minus GlcNAc ($[MH_2]^{2+}$ = 1411.1 *m/z*).

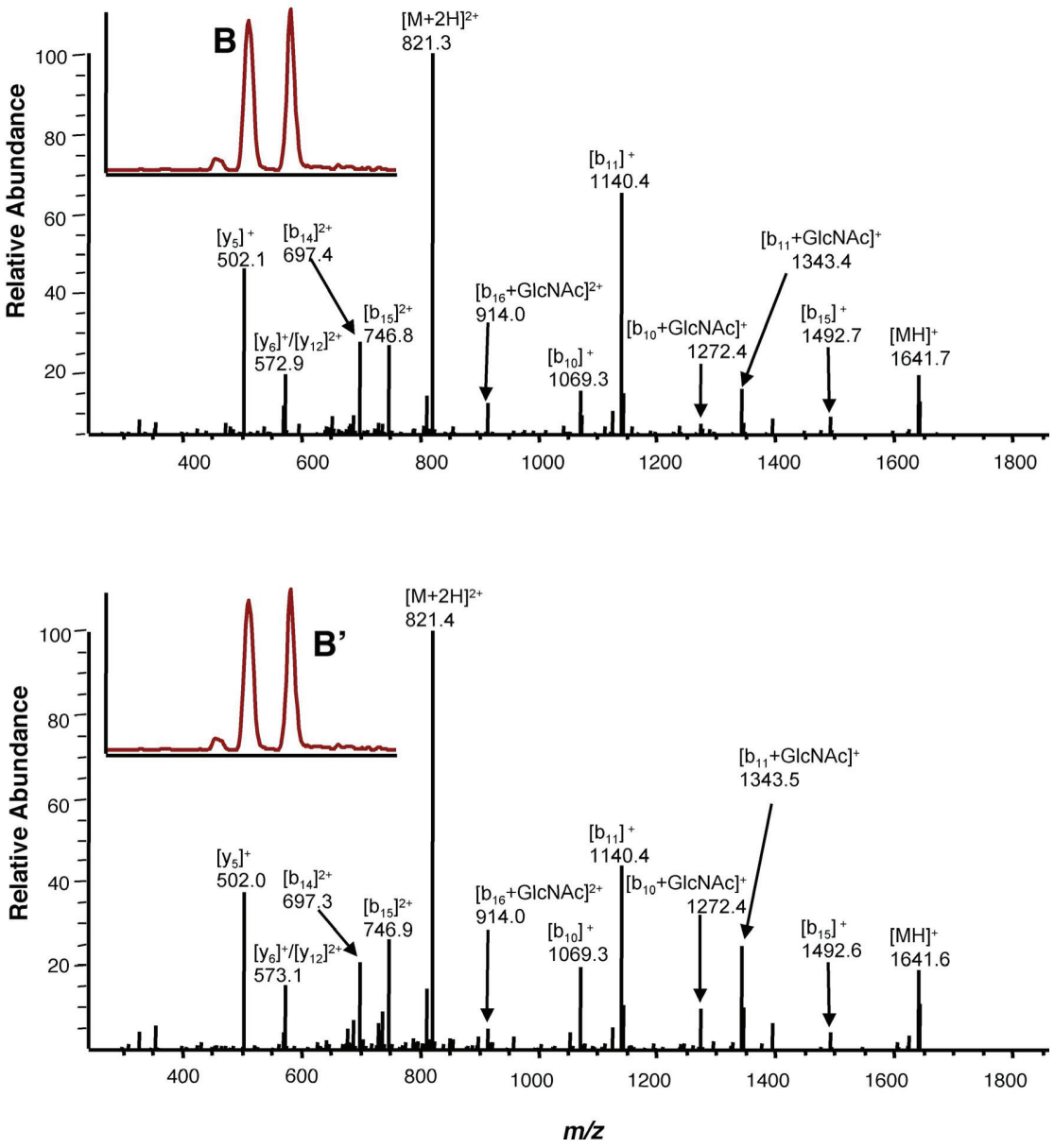


Appendix Figure 3. LC-MS/MS analysis of recombinant CREB co-expressed with OGT. **(A)** Selective ion chromatograms reveal two distinct monoglycosylated forms of a tryptic CREB peptide (D and D'; $[M+GlcNAc]^{2+}$ = 1513 *m/z*). **(B)** Representative tandem mass spectra of the monoglycosylated peptides D and D'. The most intense ion in the CID spectrum (1411.1 *m/z*) represents the parent ion minus GlcNAc. M = ^{256}TAPTSTIAPGVVMASSPALPTQPAEEAAR^{284}.

Appendix Figure 4 shows selective ion chromatograms and representative MS/MS spectra for the monoglycosylated peptides obtained from chymotryptic digestion of recombinant or native CREB, respectively. For native CREB, LC-MS experiments were run in single ion monitoring (SIM) mode with selected parent ions: 1024.5 *m/z* ($[MH_2+2xGlcNAc]^{2+}$, where M = ^{253}QIRTAPTSTIAPGVVM^{268}), 922.8 *m/z* ($[MH_2+GlcNAc]^{2+}$) and 821.5 *m/z* ($[M+2H]^{2+}$). The presence of two $[MH_2+GlcNAc]^{2+}$ species (922.9 *m/z*) that produce $[MH_2]^{2+}$ fragments (821.5 *m/z*) confirmed their assignment as monoglycosylated forms of the CREB peptide (Appendix Fig. 4A). After confirmation of the identity of the monoglycosylated Q2 domain peptide, CREB submitted to labeling by OGT *in vitro* was analyzed by MALDI-ToF and LC-MS analysis. The glycosylation was not observed in the MALDI spectra, however upon further investigation using LC-MS analysis, with the mass spectrometer running in single reaction monitoring mode, two doubly charged parent ions ($[MH_2+GlcNAc]^{2+}$ = 1513.4 *m/z*) were detected and produced the unglycosylated peptide ($[MH_2]^{2+}$ = 1411.1 *m/z*) upon CID fragmentation (Appendix Fig. 5). Appendix Table 1 shows all of the peptide fragments observed by LC-MS/MS for each of the four forms.



Appendix Figure 4. Reverse phase LC-MS analysis of CREB co-expressed with OGT. Selective ion chromatograms reveal four distinct forms of a chymotryptic CREB peptide. Diglycosylated peptide (A) eluted first from the C18 column, followed by two monoglycosylated peptides (B and B') and the unglycosylated peptide (C). M = ^{253}QIRTAPTSTIAPGVVM^{268}.



Appendix Figure 4, continued. LC-MS/MS analysis of the chymotryptic CREB ions of 922.9 *m/z* , B and B'. CID sequence analysis positively identified both peaks as monoglycosylated forms of the CREB peptide, M= ^{253}QIRTAPTSTIAPGVVM^{268}. The most intense ion in the CID spectrum (821 *m/z*) represents the parent ion minus GlcNAc.

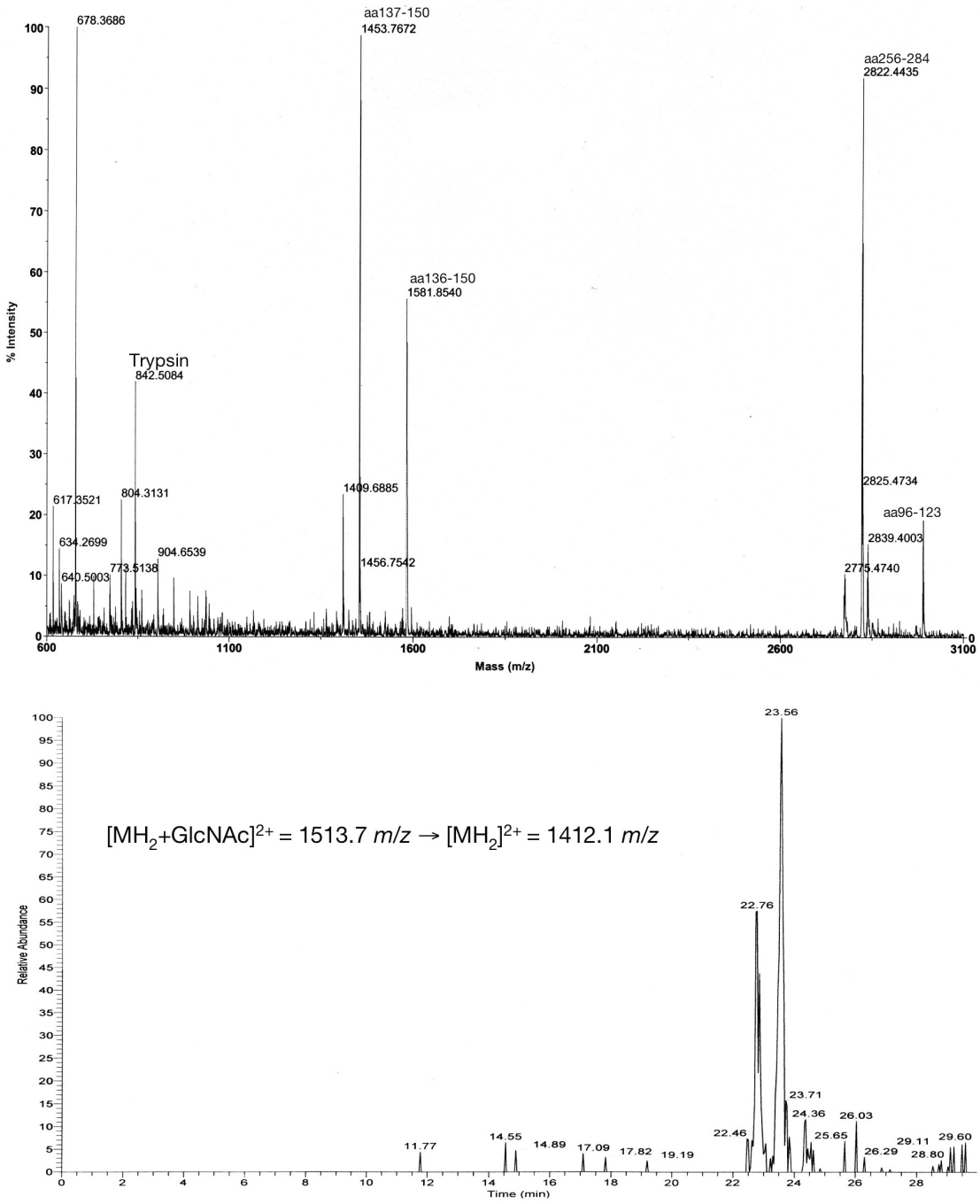
	observed m/z	calculated m/z	assignment	amino acids
A : Di-glycosylated peptide	1069.3	1069.6	$[b_{10}]^+$	QIRTAPTSTI
	1642.5	1641.9	$[M+H]^+$	QIRTAPTSTIAPGVVM
	923.7	922.9	$[M+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc
	1024.4	1024.5	$[M+GlcNAc_2]^{2+}$	QIRTAPTSTIAPGVVM + $GlcNAc_2$

	observed m/z	calculated m/z	assignment	amino acids
B : Mono-glycosylated peptide	1069.3	1069.6	$[b_{10}]^+$	QIRTAPTSTI
	1272.4	1272.7	$[b_{10}+GlcNAc]^+$	QIRTAPTSTI + GlcNAc
	1140.4	1140.6	$[b_{11}]^+$	QIRTAPTSTIA
	1343.4	1343.7	$[b_{11}+GlcNAc]^+$	QIRTAPTSTIA + GlcNAc
	1393.6	1393.8	$[b_{14}]^+$	QIRTAPTSTIAPGV
	697.4	697.8	$[b_{14}]^{2+}$	QIRTAPTSTIAPGV
	1492.7	1492.8	$[b_{15}]^+$	QIRTAPTSTIAPGVV
	746.8	747.4	$[b_{15}]^{2+}$	QIRTAPTSTIAPGVV
	914.0	914.5	$[b_{16}+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc
	502.1	502.3	$[y_5]^+$	PGVVM
	572.9	573.3	$[y_6]^+$ or $[y_{12}]^{2+}$	APGVVM
	1641.7	1641.9	$[M+H]^+$	QIRTAPTSTIAPGVVM
	821.3	821.5	$[M+2H]^{2+}$	QIRTAPTSTIAPGVVM
	923.0	923.0	$[M+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc

	observed m/z	calculated m/z	assignment	amino acids
B' : Mono-glycosylated peptide	1069.3	1069.6	$[b_{10}]^+$	QIRTAPTSTI
	1272.4	1272.7	$[b_{10}+GlcNAc]^+$	QIRTAPTSTI + GlcNAc
	1140.4	1140.6	$[b_{11}]^+$	QIRTAPTSTIA
	1343.5	1343.7	$[b_{11}+GlcNAc]^+$	QIRTAPTSTIA + GlcNAc
	1393.5	1393.8	$[b_{14}]^+$	QIRTAPTSTIAPGV
	697.3	697.8	$[b_{14}]^{2+}$	QIRTAPTSTIAPGV
	1492.6	1492.8	$[b_{15}]^+$	QIRTAPTSTIAPGVV
	746.9	747.4	$[b_{15}]^{2+}$	QIRTAPTSTIAPGVV
	914.1	914.5	$[b_{16}+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc
	502.0	502.3	$[y_5]^+$	PGVVM
	573.1	573.3	$[y_6]^+$ or $[y_{12}]^{2+}$	APGVVM
	1641.6	1641.9	$[M+H]^+$	QIRTAPTSTIAPGVVM
	821.4	821.5	$[M+2H]^{2+}$	QIRTAPTSTIAPGVVM
	922.9	923.0	$[M+GlcNAc]^{2+}$	QIRTAPTSTIAPGVVM + GlcNAc

	observed m/z	calculated m/z	assignment	amino acids
C : Unglycosylated peptide	1069.4	1069.6	$[b_{10}]^+$	QIRTAPTSTI
	1140.4	1140.6	$[b_{11}]^+$	QIRTAPTSTIA
	1393.6	1393.8	$[b_{14}]^+$	QIRTAPTSTIAPGV
	697.2	697.8	$[b_{14}]^{2+}$	QIRTAPTSTIAPGV
	746.9	747.4	$[b_{15}]^{2+}$	QIRTAPTSTIAPGVV
	812.4	813	$[b_{16}]^{2+}$	QIRTAPTSTIAPGVVM
	502.1	502.3	$[y_5]^+$	PGVVM
	573.0	573.3	$[y_6]^+$ or $[y_{12}]^{2+}$	APGVVM
	1641.6	1641.9	$[M + H]^+$	QIRTAPTSTIAPGVVM
	821.5	821.5	$[M +2H]^{2+}$	QIRTAPTSTIAPGVVM

Appendix Table 1. Summary of all fragment ions observed by LC-MS and tandem MS for CREB peptide ^{253}QIRTAPTSTIAPGVVM^{268} generated by digest with chymotrypsin.

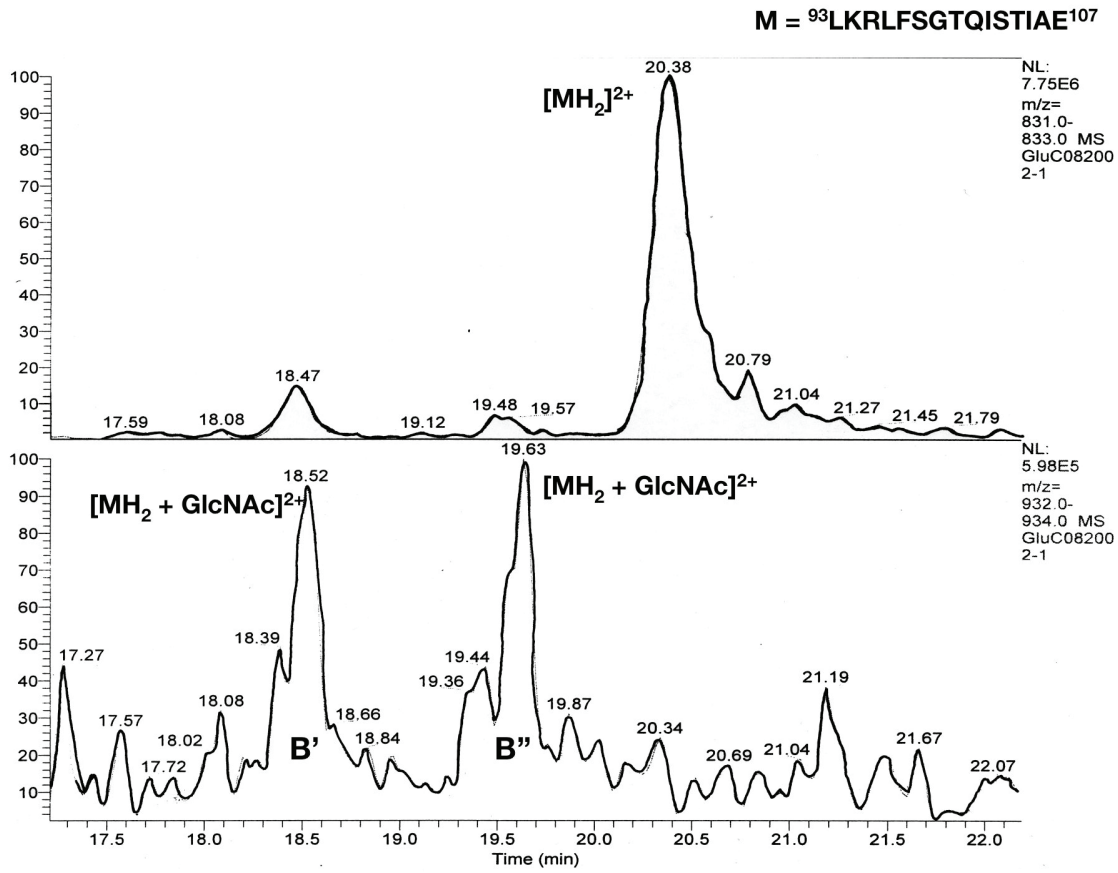


Appendix Figure 5. MALDI-ToF and LC-MS/MS analysis of recombinant CREB labeled by OGT *in vitro*. **(A)** MALDI-ToF spectra of recombinant CREB from OGT-labeling reaction showed no detectable glycosylated Q2 domain peptide (2822.4 *m/z*, M = ^{256}TAPTSTIAPGVVMASSPALPTQPAEEAAR^{284}). **(B)** Single reaction monitoring on a LC-MS instrument was used to detect deglycosylation of the mono-glycosylated Q2 domain peptide ([MH_2 + GlcNAc]$^{2+}$ = 1513.7 *m/z* → [MH_2]$^{2+}$ = 1412.1 *m/z*).

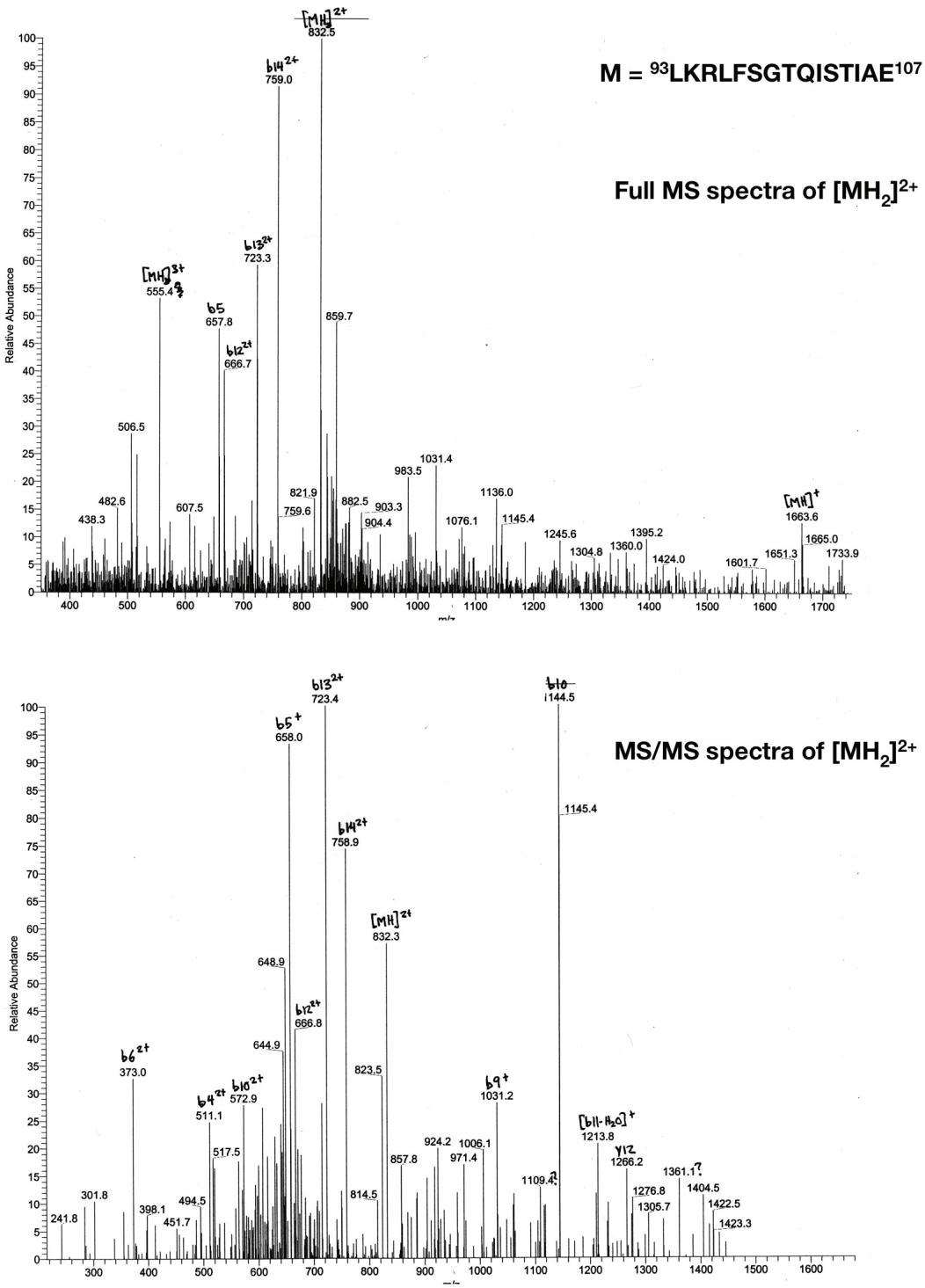
LC-MS/MS analysis of Glu-C proteolytic digest of CREB confirmed the identity of a KID domain peptide with two sites of glycosylation (Appendix Fig. 6). Samples were submitted to LC-MS/MS analysis as described above. Two monoglycosylated, 934.0 *m/z* ($[MH_2+GlcNAc]^{2+}$, where M = ^{93}LKRLFSGTQISTIAE^{107}), and an unglycosylated, 821.5 *m/z* ($[M+2H]^{2+}$), were detected in single ion chromatograms in a pattern similar to those observed for tryptic and chymotryptic digests (Appendix Fig. 6A). The MS/MS spectra of the unglycosylated peptide confirmed the assignment of the ion (Appendix Fig. 6B). Full MS spectra of monoglycosylated ions B' and B" verified their assignments (Appendix Fig. 6C).

The MS/MS spectra of the monoglycosylated forms of recombinant and native CREB are similar (Appendix Fig. 3B and 9). The native CREB showed fragment ions of weaker relative abundance due to the smaller amount of protein available for analysis.

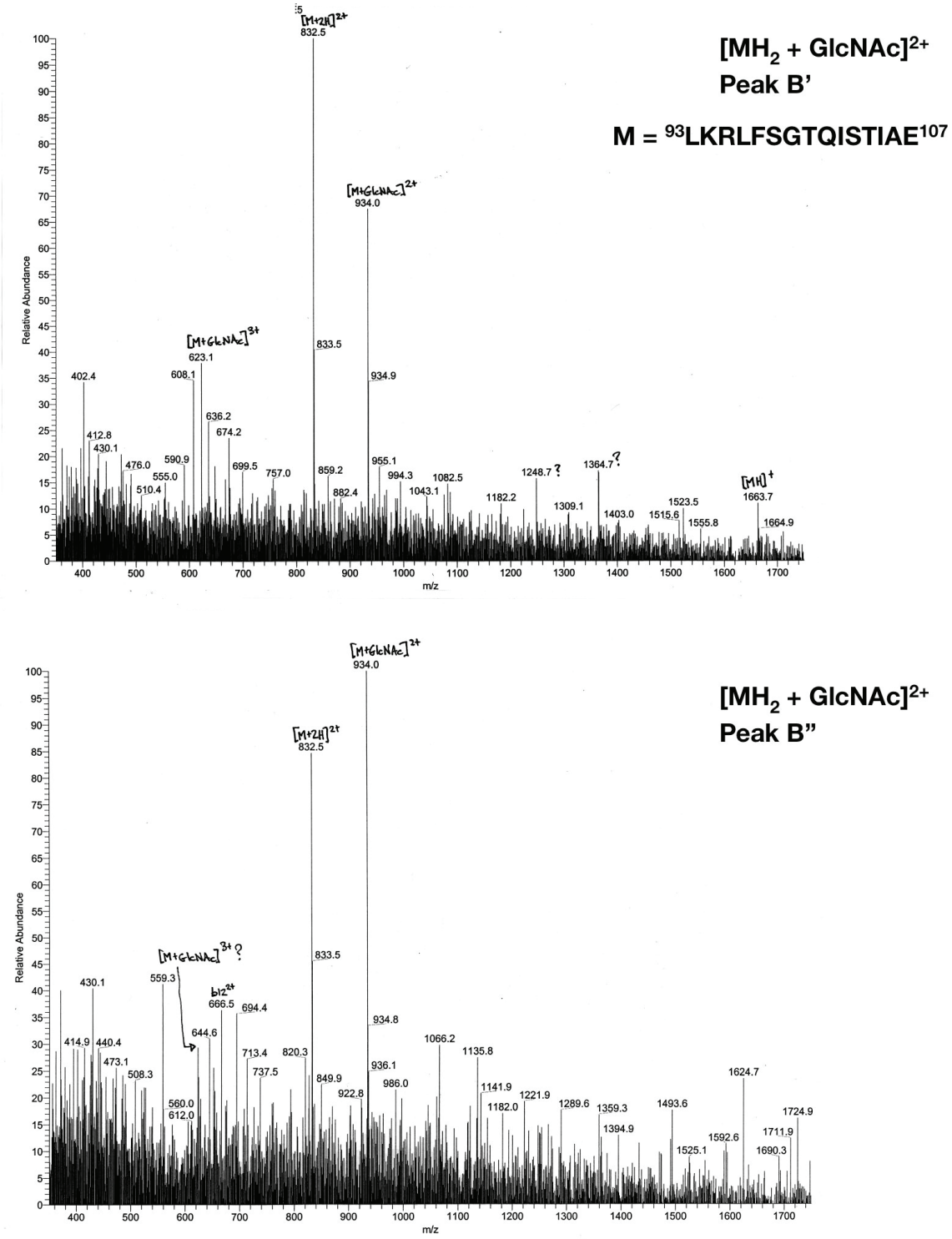
To calculate the stoichiometry of glycosylation within region 256-284, the on-line LC-MS peaks assigned to the four distinct forms of peptide 256-284 were integrated using the Xcalibur software. CREB expressed in the presence and absence of OGT exhibited 55.4% and 9.6% glycosylation, respectively. Native CREB purified from rat brain contained approximately 0.6% glycosylation.



Appendix Figure 6. LC-MS/MS analysis of recombinant CREB digested with Glu-C. **(A)** Selective ion chromatograms indicate the presence of two mono-glycosylated forms of the KID domain Glu-C peptide ($[MH_2]^{2+}$ = 832.5 m/z and $[MH_2 + GlcNAc]^{2+}$ = 934.0 m/z, M = ^{93}LKRLFSGTQISTIAE^{107}).



Appendix Figure 6, continued. LC-MS/MS analysis of recombinant CREB digested with Glu-C. **(B)** Identity of the KID domain peptide was confirmed by full MS and MS/MS spectra.



Appendix Figure 6, continued. LC-MS/MS analysis of recombinant CREB digested with Glu-C. **(C)** Identity of the mono-glycosylated KID domain peptide were confirmed by full MS spectra of peaks **B'** and **B"**.

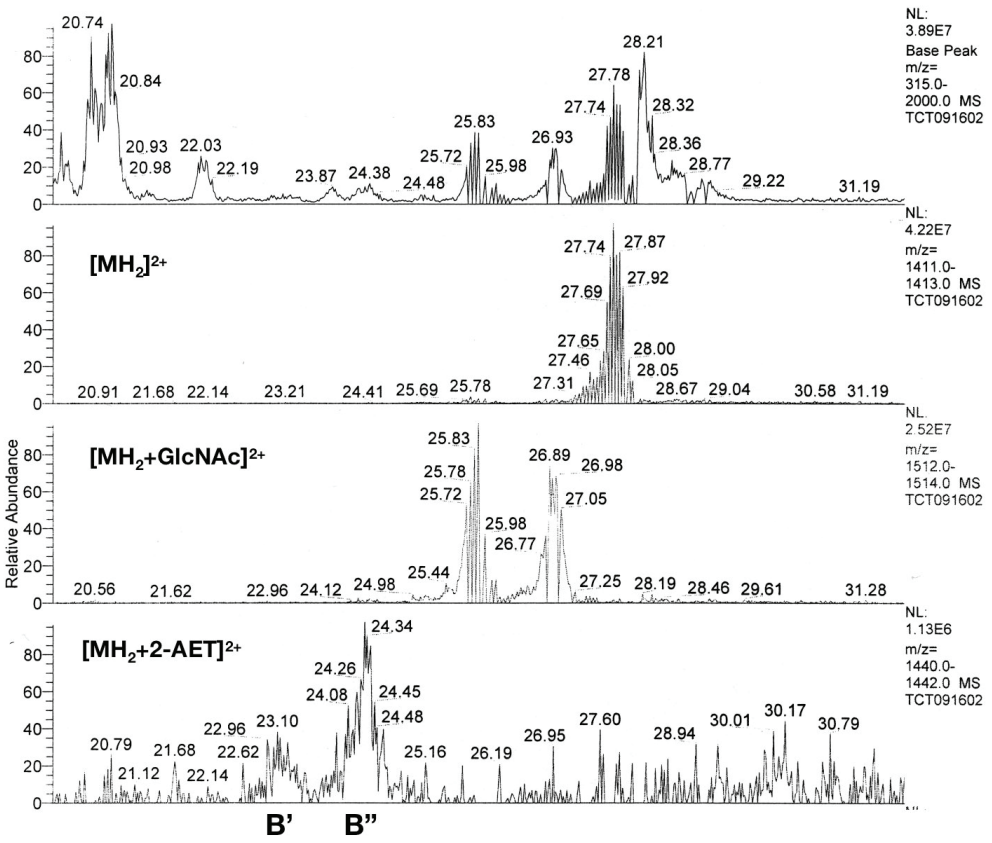
β-Elimination and Ethylenediamine and 2-Aminoethanethiol Modification.

The identification of sites of serine and threonine post-translational modification by β-elimination and modification have been described previously [7, 8]. I describe here a variation on the method *O*-GlcNAc site identification first described by Rusnak and coworkers [9]. Recombinant CREB was resolved by SDS-PAGE and submitted to proteolytic digest as described above. After proteolytic digest, peptide samples were extracted and concentrated to dryness by SpeedVac. Samples were resuspended in either ethylenediamine (50-100 μl) and incubated at 60° C for 4 h, or 0.14 M 2-aminoethanethiol 25% aqueous NH_4OH (100 μl) and incubated at 45° C for 4 h. Samples were dried under vacuum and either prepared for MALDI-ToF analysis or resubmitted to an additional round of tryptic digest as described above.

Appendix Figure 7A shows selective ion chromatograms for five distinct forms of a tryptic peptide corresponding to residues 253-268 of CREB (M= ^{256}TAPTSTIAPGVVMASSPALPTQPAEEAAR^{284}). As previously, sequential elution of the glycoforms was observed by reverse-phase LC, but with the two 2-AET-modified forms (B' and B") eluting first, followed by the monoglycosylated forms and then the unglycosylated peptide. Representative full MS and MS/MS spectra of the unglycosylated peptide verified its identity ($[MH_2]^{2+}$ = 1411.2 *m/z*) (Appendix Fig. 7B). Representative full MS and MS/MS spectra of the 2-AET modified peptides B' and B" ($[MH_2$+2-AET$]^{2+}$ = 1441.2 *m/z*) verified their identity (Appendix Fig. 7C). The identification of y25+2-AET and b8+2-AET fragment ions suggests that the sites of glycosylation reside at Ser260 and/or Thr261.

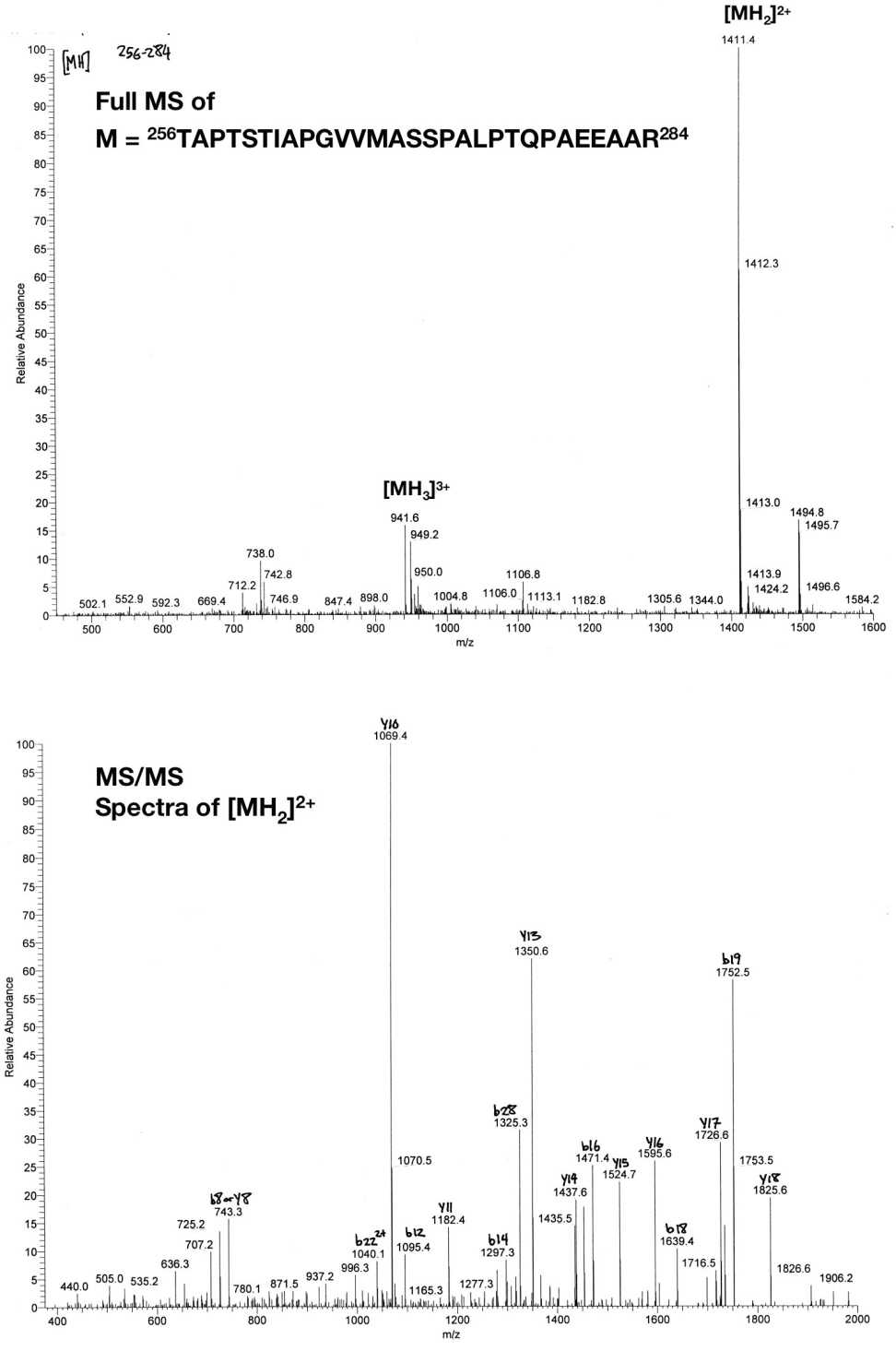
Appendix Figure 8 shows representative MALDI-ToF spectra of a tryptic CREB digest before treatment and after 2-AET treatment and a second round of trypsin treatment. In the MALDI-ToF spectra of the trypsin/2-AET/trypsin digest, the emergence of a novel tryptic peptide at 2364.9 *m/z* corresponds to the conversion of Ser260 to 4-azalysine to generate the tryptic fragment M= ^{261}TIAPGVVMASSPALPTQPAEEAAR^{284} ($[MH]^+$ = 2364.7 *m/z*).

A

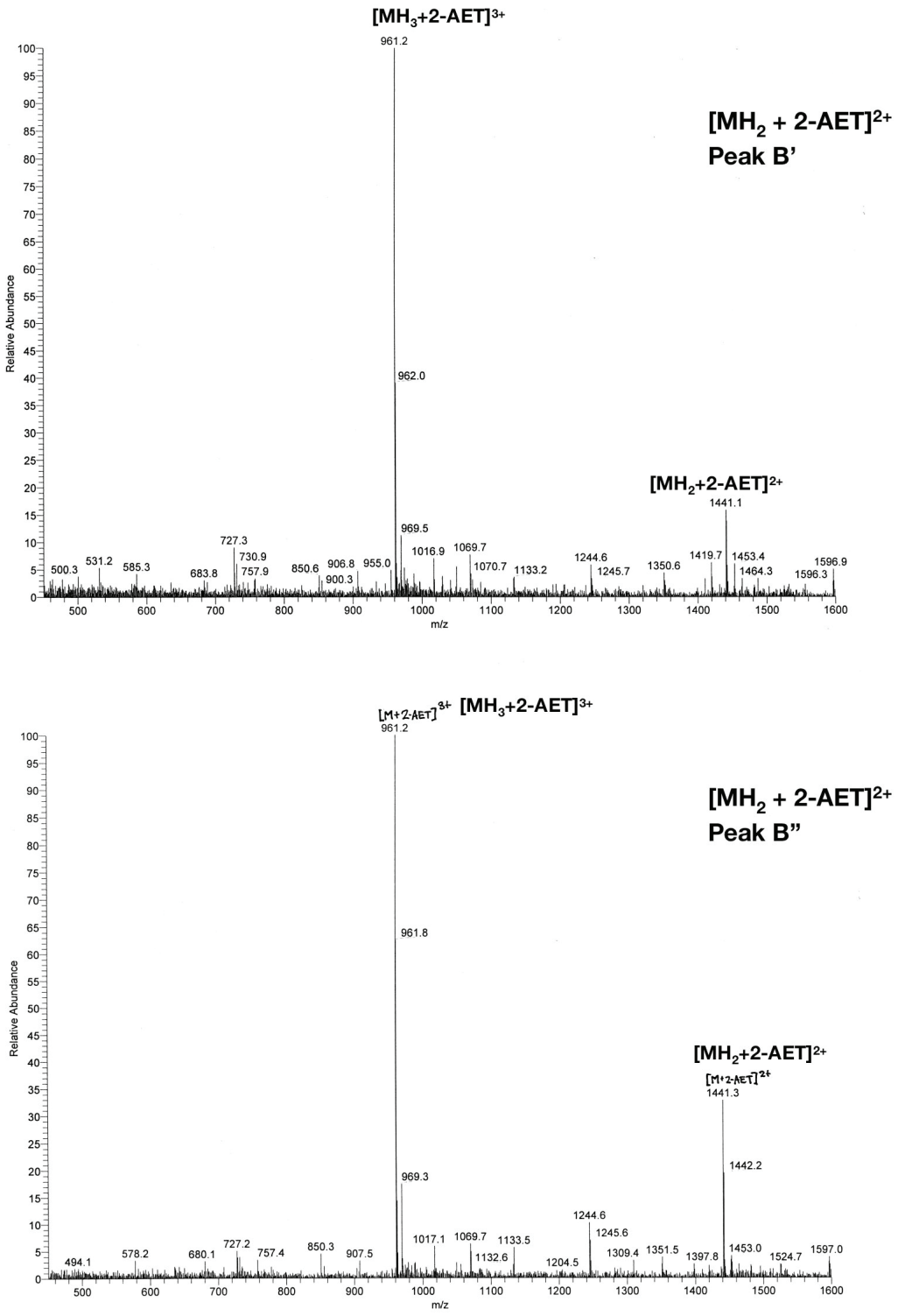


Appendix Figure 7. LC-MS/MS analysis of trypsin digest, 2-aminoethanethiol (2-AET) treatment of recombinant CREB with high glycosylation stoichiometry. **(A)** Selective ion chromatograms showing the sequential elution of $[MH_2+2\text{-AET}]^{2+}$, $[MH_2+GlcNAc]^{2+}$ and $[MH_2]^{2+}$ peptides (M=QIRTAPTSTIAPGMMASSPALPTQPAEEAAR).

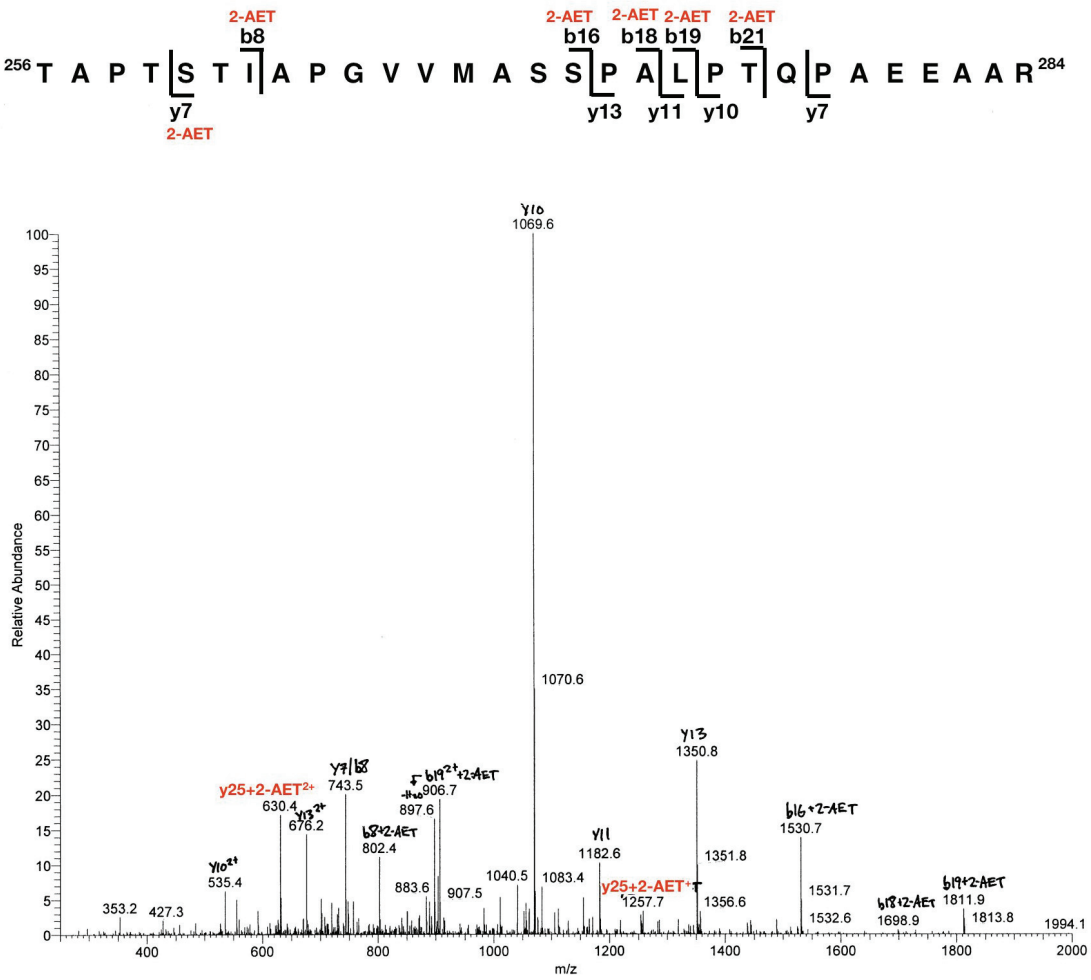
B



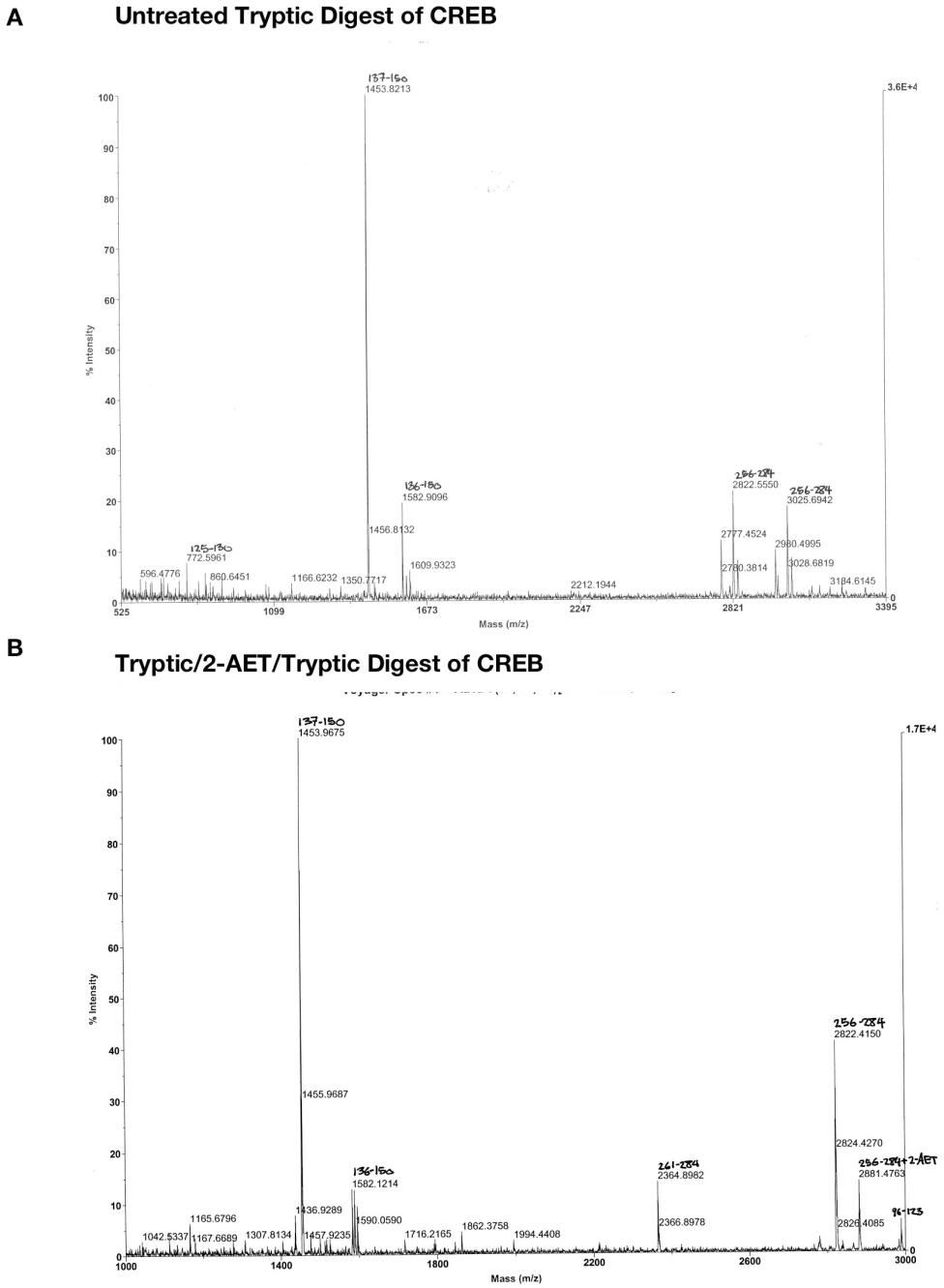
Appendix Figure 7, continued. LC-MS/MS analysis of trypsin digest, 2-aminoethanethiol (2-AET) treatment of recombinant CREB with high glycosylation stoichiometry. **(B)** Full MS and MS/MS analysis of the unmodified peptides.



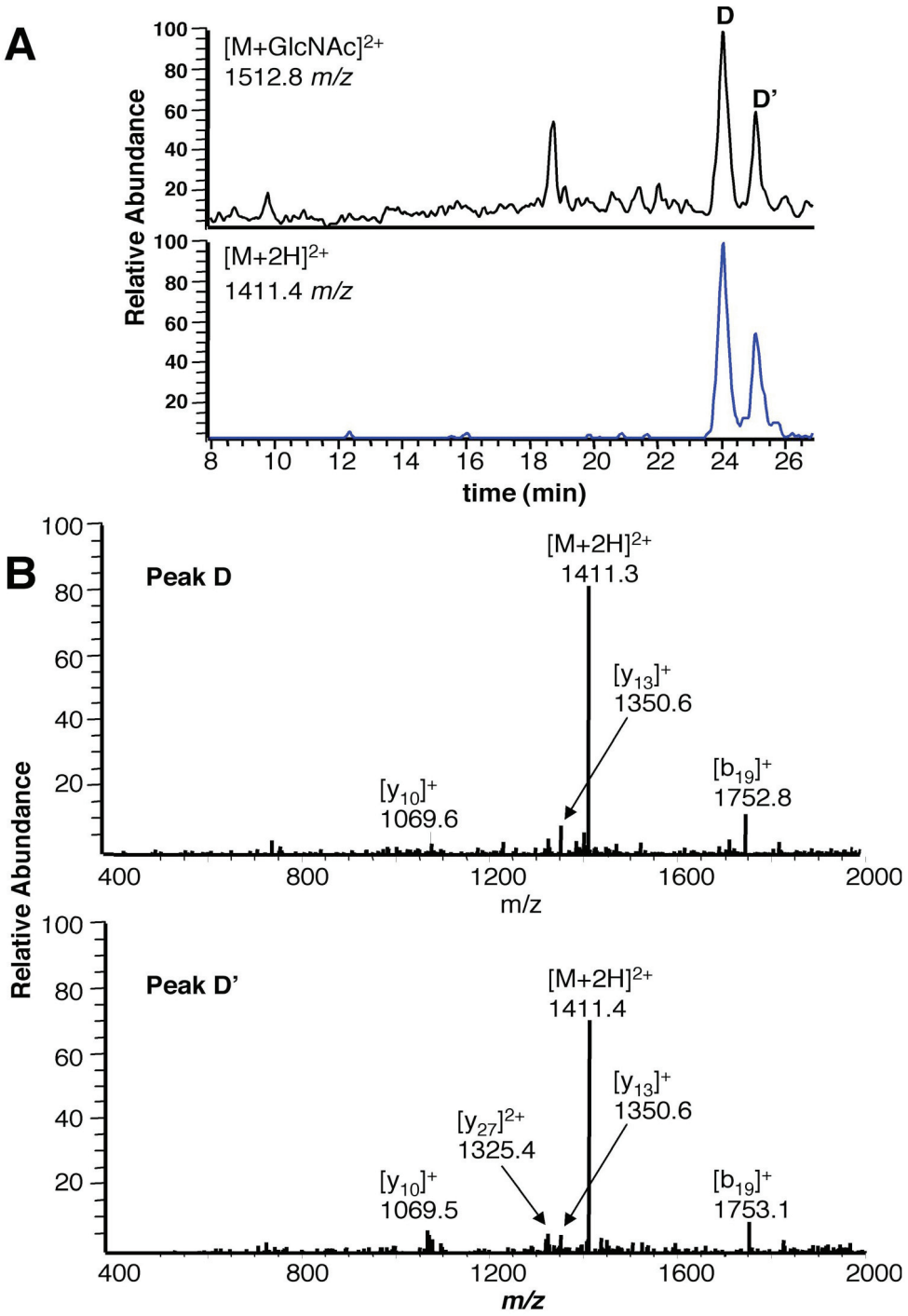
Appendix Figure 7, continued. LC-MS/MS analysis of trypsin digest, 2-aminoethanethiol (2-AET) treatment of recombinant CREB with high glycosylation stoichiometry. **(C)** Full MS and MS/MS analysis of 2-AET-modified peptides.



Appendix Figure 7, continued. LC-MS/MS analysis of trypsin digest, 2-aminoethanethiol (2-AET) treatment of recombinant CREB with high glycosylation stoichiometry. **(C)** Full MS and MS/MS analysis of 2-AET-modified peptides verify identity and potential sites of glycosylation between ^{260}ST^{261} with identification of potential y25+2-AET ion.



Appendix Figure 8. MALDI-ToF analysis of trypsin digest, 2-aminoethanethiol (2-AET) treatment and subsequent trypsin (trypsin/2-AET/trypsin) digest of recombinant CREB with high glycosylation stoichiometry. **(A)** MALDI-ToF of untreated tryptic CREB peptides.**(B)** MALDI-ToF of trypsin/2-AET/trypsin treated recombinant CREB. Generation of ^{261}TIAPGVVMASSPALPTQPAEEAAR^{284} peptide (2364.9 m/z) marks Ser260 as a site of glycosylation, β-elimination, 2-AET addition and secondary cleavage by trypsin.



Appendix Figure 9. LC-MS/MS analysis of native CREB purified from rat brain. (A) Selective ion chromatograms reveal two distinct monoglycosylated forms of a tryptic CREB peptide (D and D'; $[M+GlcNAc]^{2+}$ = 1513 *m/z*). The 1411.4 *m/z* selective ion chromatogram shows that only peaks D and D' generate daughter ions equivalent to the loss of GlcNAc. The minor difference in retention times of D and D' between recombinant and native CREB is due to the use of different HPLC columns. (B) Representative tandem mass spectra of the monoglycosylated peptides D and D'. M = ^{256}TAPTSTIAPGVVMASSPALPTQPA EEAAR^{284}.

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